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TITLE: Analysis of the Link Between Acquired Expression of a  
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Breast Cancer and Bone Metastasis

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<b>13. ABSTRACT (Maximum 200 Words)</b>  Bone metastasis of breast cancer is a major cause of death among breast cancer patients. However, we still know relatively little about why many breast cancers metastasize to the bone. To develop better treatments of bone metastasis of breast cancer, we need to understand how breast cancer cells acquire the abilities to move to the bone, survive in the new environment, and flourish as metastatic tumors. We postulate that one potential mechanism by which breast cancer cells may acquire such abilities is their acquired expression of bone specific proteins that are known to be involved in mediating the activities of the bone-forming cells in the bone tissue, the osteoblasts.  In this study, we attempted to address the critical question of whether the expression of a master gene for the development of bone-forming osteoblast cells, CBFA1, by the breast cancer cells leads to bone metastasis in an established animal model system. To do this, we manipulated the expression of this gene in established human breast cancer cell lines and planned to monitor the ability of those cancer cells to grow in the bone as metastases. A positive finding from such studies will pave the way for the development of potential therapeutic agents for the treatment of this horrifying disease.				
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## Introduction

Bone metastasis of breast cancer is a major cause of death among breast cancer patients. However, the mechanism underlying the prevailing metastasis of breast cancer to the bone remains largely unknown. To develop better treatments of bone metastasis of breast cancer, we need to understand how breast cancer cells acquire the abilities to adhere preferentially to the bone tissue, form beneficial interactions with the microenvironment of bone marrow endothelium, and re-establish angiogenesis to support the growth of metastatic colonies. One potential mechanism by which breast cancer cells may acquire such abilities is their acquired expression of bone specific proteins that are known to be involved in mediation of osteoblast activities.

In this study, we attempted to test the hypothesis that acquired expression of a master switch gene of osteoblast differentiation, *Cbfa1/Runx2*, by breast cancer cells leads to the expression of multiple bone-specific genes and acquisition of cellular activities associated with osteoblasts, leading to selective adaptation by the breast cancer cells of a tumor phenotype favorable for the homing, adhesion, and establishment of metastatic colonies in the bone.

## Body

*Task 1:* To determine the effect of repression of *Cbfa1* expression in breast cancer cells on bone metastasis

- a. Develop cell populations or individual clones of MDA-MB-231 cells with reduction or elimination of *Cbfa1* expression through stable introduction of RNAi construct by retroviral delivery

At the start of this project, we found that the MDA-MB-231 cells represent a heterogeneous population. To achieve the goal of determining if there is a relationship between the expression levels of *Cbfa1* and the functional consequence on the ability of those cells to metastasize to the bone, we isolated a total of more than 30 individual cell clones from the parental MDA-MB-231 cells and examined their expression of *Cbfa1* by western blot analysis. As shown in Fig. 1, the expression of *Cbfa1* appears to vary among different cell clones that are presented, although all of them express this protein. This result confirms our initial hypothesis that *Cbfa1* is expressed in this metastatic breast cancer cell line that has an epithelial cell origin.

Subsequently, we determined if the *Cbfa1* expressed in the breast cancer cells is functionally capable of activating transcription through a consensus *Cbfa1* DNA binding element termed OSE detected in the promoters of several *Cbfa1* target genes. As shown in Fig. 2, the activity of the 6X wild type OSE driven reporter is highly elevated in several independent clones that were shown to express *Cbfa1* (Fig. 1). As a negative control, a reporter driven by 6X mutant OSE containing a point mutation in the OSE sequence showed no activity in all the clones. Interestingly, the activity of the OSE-reporter displayed various degrees among different clones, suggesting that additional layers of regulation on the transcriptional activity of *Cbfa1* may exist in the breast cancer cells.

To generate MDA-MB-231 cells with reduced *Cbfa1* expression, we have screened a number of siRNA constructs based on the pSUPER vector as the vehicle for stable delivery. To speed up the screening process to identify suitable siRNA constructs for this purpose, we

have tested the ability of transient or stable transfection of four different pSUPER-Cbfa1 constructs in human Saos-2 osteosarcoma cells that express a high level of Cbfa1. We have also tried to assess the ability of those constructs to knockdown the ectopically expressed Cbfa1 in 293 cells since the transfection efficiency is high. However, we have so far failed to identify a siRNA construct that could effectively reduce the expression of Cbfa1 using those two systems (two examples of the siRNA constructs are shown in Fig. 3). We are currently in the process of testing other methods to achieve the goal of knocking down the expression of Cbfa1 in the breast cancer cells.

- b. Perform intracardiac injection of the MDA-MB-231 cells on SCID mice to evaluate the metastatic potential to the bone

We have attempted a number of times of introducing the MDA-MB-231 cells into the immune-compromised mice via the route of intracardiac injection. So far we have encountered significant technical difficulties in successfully inoculating the cancer cells through this route. As shown in Fig. 3, we have been able to introduce the cancer cells into the animals as shown by the formation of tumors in several organs of the animals detected one month after the injection. We are still waiting to see if any osteolytic lesions appear in those animals in another two months of time (based on published literature, it typically takes about three months for the bone lesions to appear after the initial inoculation of breast cancer cells via intracardiac injection. Once we master this technique, we will assess the ability of different clones of the breast cancer cells with different levels of Cbfa1 expression to form bone lesions.

- c. Initiate biochemical and biological assays to examine the functional consequences of reduction or elimination in Cbfa1 expression at the cellular and molecular level

As shown in Fig. 2, we have started functional evaluation on the biological activity of Cbfa1 in the breast cancer cells and found that the transcriptional activity of Cbfa1 varies among different cell clones. We will next test the ability of Cbfa1 to bind to DNA sequences containing the OSE by using gel mobility shift assay.

*Task 2.* To determine the effect of ectopic expression of Cbfa1 in non-metastatic breast cancer cells on their metastatic potential to the bone

- a. Generate cell populations or individual clones of MCF-7 and T47D cells with ectopic expression of Cbfa1.

We have attempted numerous times to ectopically express Cbfa1 in MCF-7 and T47D breast cancer cells without being able to isolate any single cell clones that express the gene. All the clones were found to contain the co-introduced drug-resistant gene that was on the same DNA construct as the Cbfa1 cDNA. Those experiences prompted us to conclude that the expression of Cbfa1, a master switch gene that potently drives the osteogenic differentiation program, might not be compatible with the proliferation program of the breast cancer cells unless the genetic lesions carried by the cancer cells tolerate the presence of such a potent differentiation gene. Thus, the breast cancer cells capable of expressing the Cbfa1 gene and metastasizing to the bone must contain unique sets of genetic alterations that distinguish them from those breast cancer cells that are incapable of forming bone metastasis, such as the MCF7 and T47D

cells. We believe that this concept should be systemically tested in the future.

- b. Test the effect of Cbfa1 expression on bone metastatic potential by the two types of cells.

As stated above, we were unable to perform these experiments due to the failure of generating stable cell clones expressing Cbfa1.

- c. Initiate biochemical and biological assays to examine the functional consequences of Cbfa1 expression at the cellular and molecular level

The same reason as stated above for b.

### **Key research accomplishments**

1. The preliminary findings support our hypothesis that expression of Cbfa1 by breast cancer cells may be associated with bone metastasis.
2. The exploration of this idea has opened up a new line of research for studying the mechanism associated with breast cancer bone metastasis.

### **Reportable outcomes**

There are no reportable outcomes because the experiments are still in the process of generating publishable data.

### **Conclusion**

In this final report, we have summarized our research accomplishment in exploring the idea that the expression of a master osteogenic differentiation switch gene, Cbfa1, is associated with the ability of breast cancer cells to metastasize to bone. Future progress in this research direction will provide novel insight into the mechanistic basis for breast cancer bone metastasis and valuable information for the potential therapeutic application in halting cancer progression.

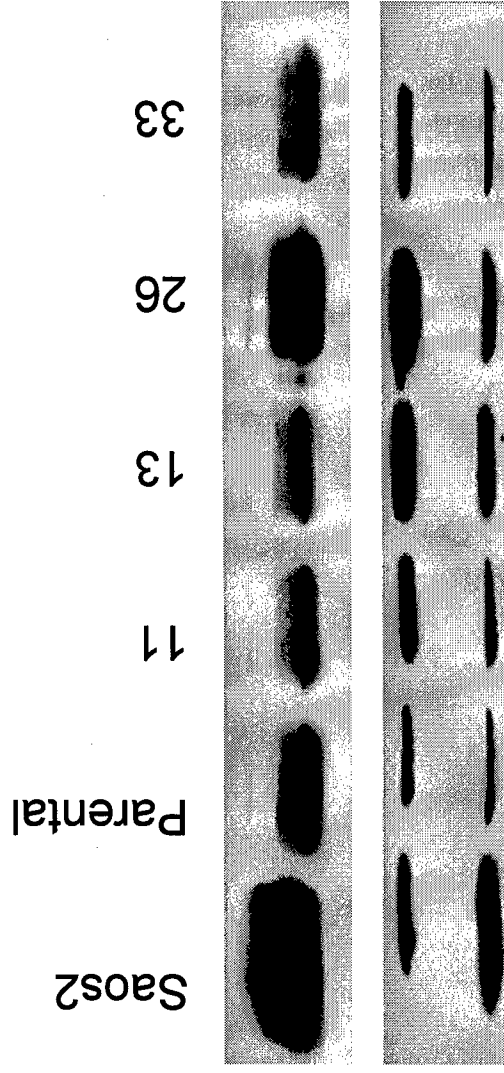
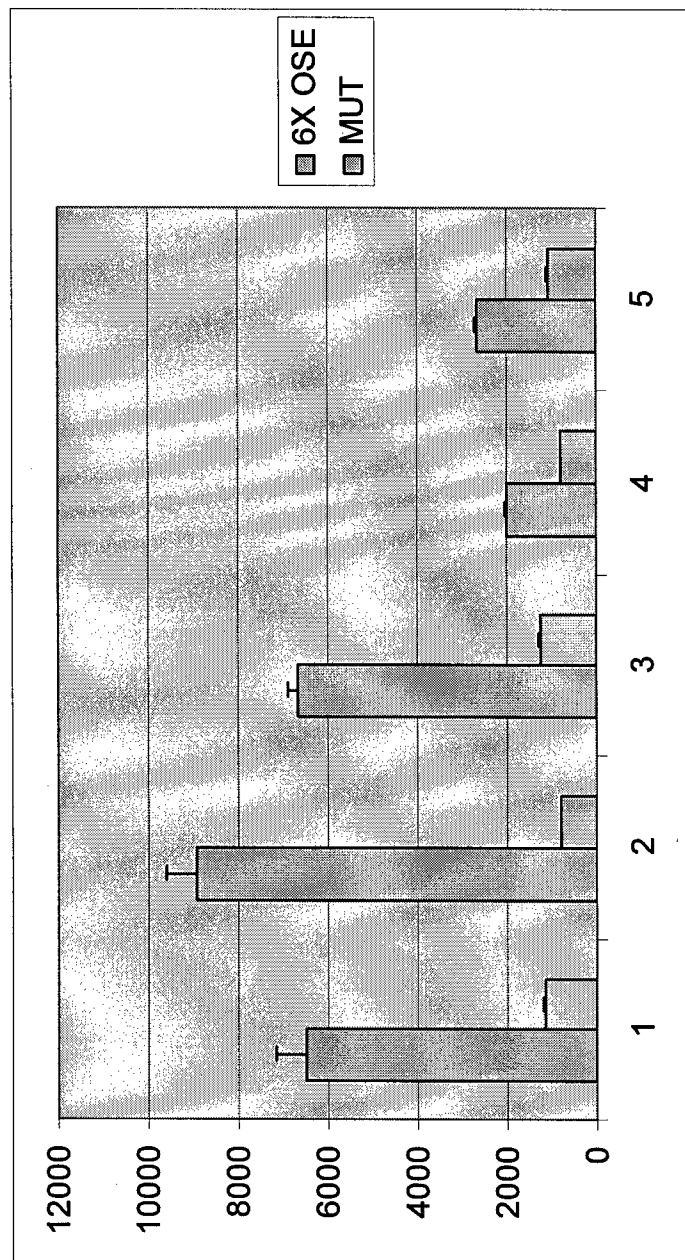


Figure 1: Cbfa1/Runx2 expression in single cell clones. Western blot analysis of Cbfa1 protein content in the parental and four independent stable cell clones established from parental MDA-MB-231 breast cancer cells. Blots were then stripped and probed with Lamin A/C antibody as a loading control. The lysate from Saos2, a human osteosarcoma cell line, is used as a positive control for the Cbfa1 antibody.



Par. 11 13 26 33

Figure 2: Cbfa1 transcriptional activity assayed on the OSE consensus promoter-driven reporter. Green bars denote the activity of 6X wild type OSE driven reporter. Yellow bars denote 6X mutant OSE driven reporter used as a negative control for the Cbfa1 activity. The parental (Par) and four independent stable MDA-MB-231 cell clones are shown.



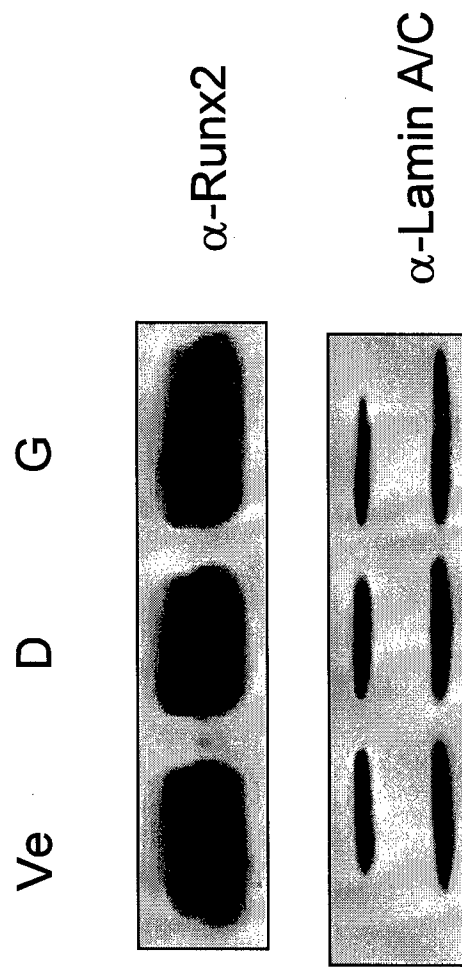


Figure 3. RNAi knockdown of Cbfa1 in Saos2 cells. Western blot analysis of Cbfa1 protein levels after transfection with pSUPER siRNA constructs (represented by two constructs, D and G). *Ve*, vector alone. Blots were subsequently stripped and re-probed with Lamin A/C antibody as a loading control.

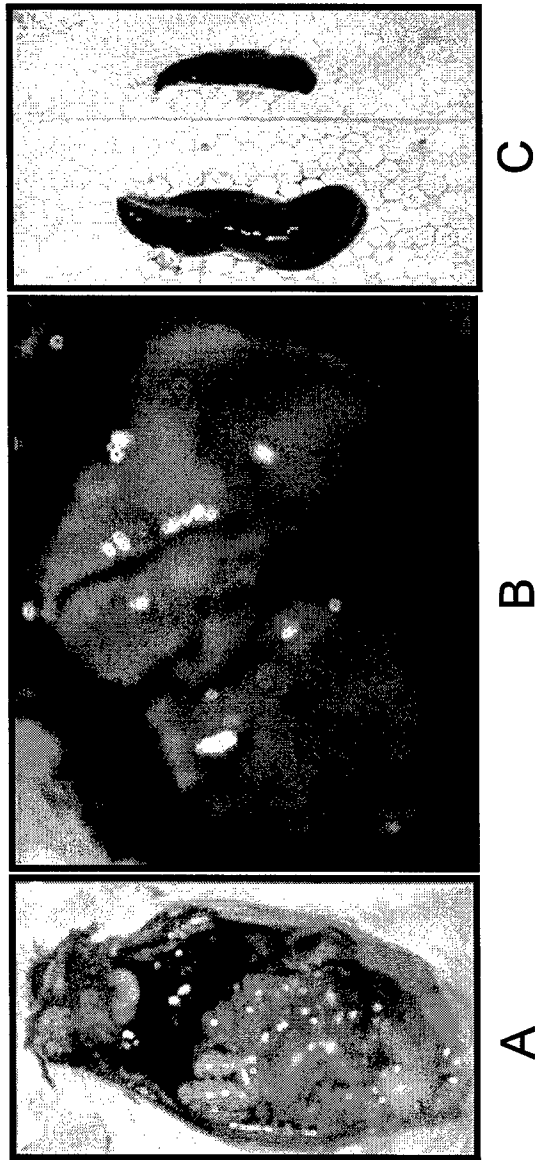


Figure 4: Intracardiac injections of MDA-MB-231 cells into immunocompromised mice. A, control mouse exhibiting no tumors. B, experimental mouse with lung metastases. C, experimental mouse with enlarged spleen (right) when compared to normal spleen (left). Spleen also exhibits tiny white nodules implicating tumor formation. All were collected one month after inoculation of the breast cancer cells.